Dr. C. W. Daniels

Protozoological Dept.

No. 31.

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SCIENTIFIC MEMOIRS

BY

OFFICERS OF THE MEDICAL AND SANITARY DEPARTMENTS

OF THE

GOVERNMENT OF INDIA

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SECOND REPORT

BY

CAPTAIN W. S. PATTON, M.B., I.M.S.
(*On special duty.*)

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BY THE SANITARY COMMISSIONER WITH THE GOVERNMENT
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THE DEVELOPMENT OF THE LEISHMAN-DONOVAN PARASITE IN CIMEX ROTUNDATUS.

SECOND REPORT.

IN my first report[1] I gave a short account of the researches of different investigators on the extracorporeal stage of the Leishman-Donovan body, and examined the hypotheses advanced to explain the method of infection of the human body by this parasite, pointing out that the disease is most probably transmitted by the bite of some blood-sucking insect. In support of this hypothesis it was shown that Kala Azar is usually contracted by people living in close contact with others suffering from the disease; that the parasites occur in the peripheral circulation in a suitable condition for further development and that in those cases with extensive ulceration of the large intestine they are often found in large numbers.

In order to prove the truth of this theory a number of feeding experiments were carried out with the common blood-sucking insects of Madras, and at the outset it was shown that the parasite could be found in the midguts of lice, *Pediculus capitis*, fed on patients suffering from Kala Azar, but no changes towards development were observed. In the bed-bug, *Cimex rotundatus*,* however, the parasites were not only frequently found but had in a few instances shown evidences of considerable development.

Since writing my first report I have been able to study in the bed-bug all the intermediate stages of the development of the parasite up to the formation of the long free swimming flagellates, and in the present paper I propose to describe these changes and some further observations on the peripheral blood of cases of Kala Azar.

I have had the opportunity of examining three more patients in whose peripheral blood parasites occurred in large numbers. In each case diarrhœa was a prominent symptom. The last patient was a man aged 25, a carpenter by trade, whose illness began in August 1906 with an attack of fever of a continuous type lasting about 14 days. During the month of September he occasionally had fever, but was able to continue his work till the first week in October. He then noticed that his feet were beginning to swell and after a short time he was obliged to give up his work owing to the œdema having spread to his ankles and legs. He was treated as an outpatient in the General Hospital, Madras, during November and December. In the first week of December he began to get diarrhœa and his relatives noticed that the right side of his face was swollen, and as his condition was much worse and the œdema had become general he was admitted to hospital on the 18th.

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* *Cimex macrocephalus* Fieb, was first described by Signoret[2] in 1852 from the Island of Réunion. I have recently adopted the name *Cimex rotundatus*,[3] which was originally given it by Signoret.

On admission, the patient was suffering from general œdema, his face was swollen, the right side being more prominent owing to a hard brawny swelling involving the whole cheek. On examining his mouth no ulcer was seen, there was marked *pyorrhœa alveolaris* and his breath had an offensive smell. On palpating his abdomen the spleen was found to be five inches below the costal margin, but the liver could not be felt. He complained of great pain over the region of the large intestine and there was evidence of a quantity of fluid in his peritoneal sac. His scrotum was also distended with œdema. On auscultating his heart there was no sign of valvular disease though the sounds in all the areas were muffled; both lungs were œdematous and there were signs of consolidation at the right base. His pulse rate was 86, his temperature 97°F. and his urine on being tested showed no trace of albumen. On December the 23rd the patient was much worse, the swelling on the right side of the face having considerably increased, involving the right eye and the upper and lower lips; and the skin over the most prominent part was glistening and suggested the early formation of an abcess. On this day I examined a film of finger blood and found twenty-three parasites, all of which were in the polymorphonuclear cells. On the 26th the patient was in a semi-conscious condition and passed his motions under him in bed; his pulse rate was 126 and the bases of both lungs were consolidated. On the 27th there was a marked change for the worse, his face was enormously swollen, the right eye being closed while the left was only just open. The skin over the swelling of the cheek was not much changed and there was no appearance of ulceration in the mouth. I took a film of finger blood and fed some bugs half an hour before the patient's death, which took place at 10-30 P.M. on the 27th. Next day on examining this film I found 359 parasites, almost every polymorphonuclear cell containing one or more. Plate I, fig. 1, shows a polymorphonuclear cell containing six parasites and Fig. 3 on the same plate an eosinophyl cell containing two; in this film I also found a large cell (Plate I, fig. 2) containing forty-nine parasites and a large mononuclear cell (Fig. 6) containing ten. In the large cell one of the parasites is seen to be dividing longitudinally; it is oval in shape measuring 4·5μ and contains two macronuclei at one pole and two micronuclei lying at opposite sides of the periphery; its protoplasm stained faintly blue and had three vacuoles in it. The close apposition to each other of many of the parasites in this cell suggests recent division of the parasites. A similar cell (Plate I, fig. 4) was found in a film of finger blood taken from the second patient two days before his death. This cell contains thirty-two parasites six of which are seen lying superimposed on the nucleus ; its protoplasm stained dark blue with Romanowsky's stain and was much vacuolated.

Christophers[4] had previously found similar cells in films made from the blood in large veins, femoral, portal, and hepatic, some hours after death ; and from a study of sections of the spleen and liver of cases of Kala Azar he concluded that these cells are derived from the vascular endothelium and that they represent the final stages of an endothelial cell which has become distended and modified by the presence of a large number of parasites. He was able to trace every gradation of flattened endothelial cell containing only a few parasites to the enormous cells almost blocking the capillaries in which they lie.

Statham[5] who has studied the distribution of the Leishman-Donovan body in the tissues confirmed these observations of Christophers and pointed out that these swollen endothelial cells are met with in the liver, spleen, bone marrow and lymphatic glands in toxæmic affections and hyperplasias of these organs ; the researches of Werigo,[6] Gilbert and Carnot[7] and others have shown that these vascular endothelial cells are phagocytic in character and that they take up bacteria and malarial pigment.

Marchand and Ledingham,[8] who also noted the occurrence of the parasites in these large cells, consider that only some originate from the vascular endothelium and that the majority are in reality enlarged splenic cells which have taken on a phagocytic action. It is of interest to note that these large endothelial cells occur in the peripheral circulation of cases of Kala Azar some days before death, and it seems most probable that the rupture of these cells and the liberation of the parasites they contain, explains the occurrence of the large numbers of parasites in the peripheral blood of patients suffering from ulceration of the large intestine. Once the parasites are set free in the plasma they are immediately taken up by mononuclear, eosinophyl or polymorphonuclear cells.

The presence of these large cells containing numerous parasites in the circulating blood further suggests the mode of formation of the brawny swellings which often develop in the later stages of the disease. Captain Long, I.M.S., first drew my attention to a patient in his ward who developed such a swelling on the lower part of the left leg. This patient had many parasites in his peripheral blood and was at the time suffering from diarrhœa. A painful swelling about 3 inches in circumference involving the skin and subcutaneous tissue was noticed on the outer side of his left leg. It gradually increased in size becoming a fluctuating tumour, and was thought to contain pus but, on being incised, sanious fluid alone exuded. Films of this fluid stained by Romanowsky's method and carefully examined showed no parasites or bacteria. I have examined many of these swellings which form in such situations as the face, back of the neck, chest, gluteal region, thighs, legs, shoulders or ischio-rectal fossa. When on the cheek they may result in an abscess opening externally or, as is more frequently the case, develop into *cancrum oris*. Another patient under observation in Captain Long's ward suddenly developed, during the later stages of the disease, a swelling in the right cheek, which in a few days resulted in a large sloughing ulcer in the mouth and later spreading outwards to the skin destroyed all the soft tissues up to the patient's right eye.

The earliest changes towards the formation of *cancrum oris* appear therefore to be identical with the brawny swellings seen in other parts of the body, and it seems probable that they are produced by a hæmorrhage caused by the blocking of a small vessel by one or more of the large cells. It can be readily understood that the resulting swellings would be liable to be invaded by bacteria and to develop into ulcers. The sudden appearance of these swellings also suggests that this explanation of their formation is the probable one.

In the film taken from the patient just before death there were some parasites showing the earliest changes towards development, and I have on many occasions seen these appearances in films of peripheral blood. Statham[5] has also noticed partial development of the parasites in smears taken from the organs of a case of Kala Azar 12 hours after death. The parasites measure from 4μ to 5μ in length, their protoplasm stains a distinct blue with Romanowsky's stain and often contains three or more vacuoles; the macronuclei, also enlarged and stained less deeply, are seen growing towards the centre of the parasites. Two cells showing these appearances are figured in a large mononuclear leucocyte in Plate I, fig. 6.

In the same film there was a large body (Plate I, fig. 5) measuring 14μ in length containing two parasites. This structure which stained deep blue by Giemsa's method was of a granular nature and appeared to be similar to bodies seen in films of splenic blood, in which situation they often contain four or more parasites. There is considerable difference of opinion as to the true nature of these bodies; Laveran,[9] Mesnil[9] and Donovan[10] consider they are red blood corpuscles altered by the presence of the parasite. Christophers[11] who studied and described the various appearances assumed by these bodies states they are detached buds of mononuclear cells or macrophages containing parasites. Treutlein[12] examining some of Rogers' preparations has recently stated he has seen the parasites in red cells, but no other observer as far as I am aware has recently described the parasites as occurring in the red corpuscles. All investigators who have studied the parasites in the human body are agreed that in sections of the organs they are never seen in the red cells.

The Development of the Parasite in the Bed-Bug.

On December 27th, twelve bugs were fed between 9 and 10 P.M. on the patient mentioned above, and on the 31st one male and three adult females were dissected between 8 A.M. and 12 noon, the midguts and their contents being smeared out on slides and stained by Giemsa's method. In these films parasites were found in an unchanged condition (Plate I, fig. 7 *a*) and in all stages of development up to the mature flagellates. The leucocytes were seen as indistinct granular masses, with or without a central deeper stained area, the remains of the nucleus; such disintegrating cells may sometimes be seen in the midgut as late as the fourth day, but as a rule they disappear by the end of the third.

The parasites are ingested by the bug while lying in the polymorphonuclear, eosinphyl, large mononuclear, and endothelial cells, and from a number of earlier observations it appears that the majority remain unchanged until the second day. While still lying in the disintegrating leucocytes they begin to show the earliest signs of development; the protoplasm increases in volume, is more granular and instead of staining a faint pink with Giemsa's stain, now appears blue throughout and contains either one large central vacuole (Plate I fig.7 *b*) or a number of smaller ones (Plate I, figs. 7 *d, e, f*).

Coincident with these changes the macronucleus becomes enlarged and stains less deeply; its central dark chromosome divides into a number of rods which are distributed throughout its substance. It may now be either circular in shape or oval with one end more pointed, and may lie at the periphery or across the centre of the parasite. The micronucleus, still rod like and staining almost black, increases in size and is usually seen lying towards the periphery of the cell. Parasites more advanced towards development have a very characteristic appearance; their protoplasm has still further increased in amount so that the body of the parasite is made up of granular blue staining protoplasm. The vacuoles also appear more distinct measuring from $\cdot 8\mu$ to 1μ in diameter; they are usually circular in shape and vary in number from four to eight. When deeply stained by Giemsa's method the periphery of these parasites which stains a much darker blue than the rest of the cell suggests a condensed layer of protoplasm of the nature of a cuticle. Parasites exhibiting these changes vary in size from 4μ to 7μ; some are oval in shape but the majority are round. In a few the macronuclei have still further increased in size and show the earliest changes towards division (Plate I, fig. 7 *f*). A constriction forms on the inner side and gradually deepens; at the same time the chromosomes begin to pass to the two ends which become rounded off, and on a similar constriction forming on the opposite side the appearance of a dumb-bell is produced (Plate I, fig. 7 *c* and fig. 8 *f*), and later two daughter nuclei are formed (Plate I, fig. 9 *f*).

Plate I, fig. 7 *c*, shows five parasites which have been lying in one leucocyte, and which, as this has degenerated, have become grouped together; one of the parasites is still in an early stage of development while the others are much further advanced. Vacuolation at this stage and later is a marked feature of the development in the bug, many of the parasites containing four or five vacuoles in close apposition but separated by strands of dark protoplasm.

From a careful study of the films it was seen that after the early stages described above one or other of two things takes place: (1) the parasites may rapidly pass on to flagellation; or (2) what is more important, they further enlarge and by consecutive division of the macro-and micronuclei produce a rosette of from four to eight parasites. Judging from the large number of these rosettes and groups of elongated flagellates still attached, it appears that the further increase in size and resulting formation of rosettes is the more usual change that takes place.

In the enlarged parasites an area staining bright pink and with a darker centre measuring about ·4μ develops in close relation to the micronucleus (Plate I, fig. 8 *a*, *b*, *c*, *d*, *e*, *f*). This structure which represents the early formation of what has been variously named 'vacuole-like area' (Christophers[13]), 'Flagellar vacuole' (Leishman[14]), and 'Eosin body' (Rogers[15]) rapidly increases in size and when fully developed is almost circular in shape measuring from 1μ to 3μ, according to the size of the parasite (Plate I, fig. 9 *d*). When stained deeply with Giemsa's stain it is seen to consist of a homogenous pink substance in which darker pink strands are scattered irregularly. It now passes to the surface of the parasite when it ruptures and a small pink brush begins to protrude directly from it (Plate I, fig. 9 *a* and *b*). When stained deeply and examined with a high magnification this brush is seen to consist of a number of delicate pink strands lying in a pink matrix and in suitable preparations the filaments can be traced into the vacuole to one or two dark pink dots in the neighbourhood of the micronucleus; but in none of the specimens was it directly attached to this structure (Plate I, fig. 9 *b*, *c*, *e* and *f*). The protrusion of the brush of filaments progresses rapidly and is seen in all stages from a relatively short curved rod (Plate I, fig. 9 *c*, *e* and *f*) to the long fully developed wavy flagellum measuring from 15μ to 20μ (Plate II, fig. 1 *a*).

From a careful study of the formation of the flagellum I believe that it forms within a vacuole and that the pink area seen when parasites are stained by Giemsa's method is due to the deposition of colouring matter in the vacuole. The fact that after the extrusion of the flagellum the vacuole collapses and then appears as a pink band on either side of the insertion of the flagellum further suggests its relation to the flagellum, and I therefore propose adopting the name 'flagellar vacuole' given it by Leishman.

Many of the round or oval parasites are seen in all the stages of flagellation while lying side by side and in some the division of the macronucleus has already taken place (Plate I, fig. 9*f*). In some of these parasites the flagellum was seen to end in an achromatic zone close to the micronucleus and Plate II, fig. 1 *a*, shows a parasite in which the flagellum passed a little beyond the micronucleus to end in a pink dot.

MacNeal[16] who has studied the attachment of the flagellum of *Trypanosoma lewisi* states that in favourable specimens it can be traced to an achromatic space in close proximity to the micronucleus; Laveran and Mesnil[1] have also noted this appearance in the same trypanosome.

Considerable variation is met with in the further stages of the first type of development described. In many parasites, as has been mentioned above before flagellation has begun, the macronucleus divides into two, and this is followed by the enlargement and division of the micronucleus. Two flagellar vacuoles develop close to the micronuclei and in some cases are seen fused together; the two flagella rapidly pass out of them and as division proceeds the flagellates are seen attached by their flagella alone (Plate II, fig. 1 *c*) or by both poles. In other cases the parasites increase in size and become much vacuolated, the macronucleus enlarges and shows signs of division while the micronucleus has already divided. Two flagella now develop a little distance apart and later become extruded and on completion of the division of the macronucleus the parasite splits longitudinally into two spindle-shaped cells (Plate II, fig. 1 *b*). Some of the enlarged oval flagellates also showed signs of elongating into these spindle-shaped forms (Plate II, fig. 1 *a* and *c*).

It will thus be seen that a certain percentage of the developing parasites pass on to flagellation and then divide longitudinally to form oval or spindle-shaped flagellates; there being no regularity observed in this process.

In the second type, development which results in the formation of rosettes consisting of from four to eight flagellates also follows on the early enlargement of the parasites. The macro-and micronuclei divide and pass to the opposite sides of the cell (Plate I, fig. 10), which has a globular appearance and is rich in granular protoplasm. One of these macronuclei divides again while the other may as yet remain unchanged or only show ? an increase in size; but the micronuclei during this time divide rapidly so that while there may be only two macronuclei, there may be three or more micronuclei (Plate I, fig. 10 *e*). Fig. 10 *d* and *f* shows the method of division of the micronucleus; after it has become thickened and considerably elongated it divides transversely into two, a method of division which is maintained throughout all the later stages of the development.

The division of the macro-and micronuclei and the further growth of the cell result in the formation of a body measuring from 10μ to 12μ in diameter;

its protoplasm stains reddish blue with Giemsa's stain and contains groups of vacuoles (Plate I, fig. 10). Such a cell may contain from four to eight macro- and micronuclei grouped about its centre. The flagellar vacuoles now develop close to the micronuclei and later all the flagella pass out from one side of the cell (Plate II, fig. 1 *d* and *e*). Separation of the flagellates now begins at the flagellar end and transparent lines pass up through the cell, and from four to eight fully developed flagellates become separated and swim away.

These flagellates vary from 6μ to 12μ in length and from 4μ to 5μ in breadth, their size depending on the rapidity with which the rosette breaks up; the majority are elongated and it is from further growth of these that the long free swimming forms are produced. It is important to note that equal longitudinal division of these flagellates while still attached or when free was not observed.

Plate II, fig. 3 *c*, shows a rosette in the process of division; the macronuclei are seen in the two parts undergoing further division, while distributed along the sides and periphery of the cell there are eight micronuclei and six flagella. Such a cell, it could be readily understood, might produce even more than eight flagellates. Plate II, fig. 3 *b*, also shows a small rosette of four elongated flagellates and two cells which have become separated from a rosette containing six parasites.

The oval or spindle-shaped parasites which are formed either from the round flagellates or from their further division exhibit very characteristic appearances. When stained deeply by Giemsa's stain they contain dark blue granular protoplasm and a number of vacuoles of varying size; their macronuclei, usually circular in shape and situated about the centre, stain deeply pink and contain a number of dark chromatic rods. The micronuclei generally lie across the long diameter of the cells near the macronuclei, seldom at the anterior ends (Plate II, fig. 2). This position of the micronuclei, as I shall mention later, indicates that the cells are about to undergo further division. The flagellum, which stains deep pink, consists of a number of filaments adhering closely to each other and they are inserted in a pale area close to the micronucleus; in none of the specimens was the flagellum seen attached along the side of the cell.

In these oval and spindle-shaped parasites further changes soon begin to take place; the macronucleus enlarges and spreading to the sides of the parasite occupies the whole of the centre of the cell, and its chromosomes become distributed throughout its substance. The micronucleus at the same time enlarges and later elongates, changes which are seen preparatory to division. The flagellum thickens and commences to divide. If this is relatively short the split extends throughout its length; but if it is long the basal portion alone thickens and the division then extends through about a quarter of its length, the new flagellum beginning to separate first at its anterior end (Plate II, fig. 2 *a*). In parasites showing a later stage of division the two flagella are completely separated and end close to the micronucleus. Preparatory to division the macronucleus in many of the cells shows a dumb-bell appearance and the two portions of the micronucleus which has divided transversely are seen side by side. Eventually a transparent line developes through the centre of the parasite, the micronuclei separate further, the macronucleus divides, and on the line of separation passing through the parasite two oval cells are produced (Plate II, fig. 2). If a spindle-shaped flagellate is an unusually large one, its division generally results in two smaller spindle-shaped cells, though it may produce two elongated parasites which are either seen still attached by their anterior ends or by both poles (Plate II, fig. 2 *c*). In some of the spindle-shaped cells a process of unequal longitudinal division was observed, a thinner parasite splitting off as described above (Plate II, fig. 2 *b*).

The majority of long flagellates shown in Plate II, fig. 4, are formed from the rosettes, a smaller number, however, particularly the thinner ones, may result from the division of the spindle-shaped cells. These long flagellates vary from 12μ to 20μ in length and from 4μ to 5μ in breadth, their macronuclei are oval in shape, stain dark pink and contain a number of rod shaped chromosomes. The anterior poles of the cells are rounded, their bodies exhibit undulations and end posteriorly in pointed beaks; their protoplasm stains dark blue containing a number of lighter areas but no vacuoles or chromatic granules. The flagella are thick filaments measuring from 16μ to 24μ in length; they originate in one or two chromatic dots usually close to the micronuclei and pass out of the cells through the centre of the anterior ends. The micronuclei are always seen lying transversely to the long axis of the parasite about $1{\cdot}5\ \mu$ to $2\ \mu$ from the anterior extremity. In many of the parasites the remains of the flagellar vacuoles are seen as indistinct pink areas surrounding the insertion of the flagella; in others they are represented by a pink line on either side of them. None of these flagellates were seen showing equal longitudinal division, and it appears doubtful whether this method of fission does take place in these cells.

The above were the only changes observed in the four films under discussion, and it will be seen that after lying 80 hours in the midgut of the bug some of the parasites were still unchanged, though the majority had passed through all the stages of development up to flagellation.

On January 1st two male and two female bugs were dissected between 12 noon and 1-30 P.M., contents of their midguts being smeared out on slides and stained by Giemsa's stain; the films from one female bug contained many parasites, but the others very few.

In each slide there were one or more parasites in an unchanged condition, as well as some showing the earlier changes towards development, and in the film made from the female bug there were also a number of rosettes and long free swimming flagellates. In addition to the above this film contained a number of pairs of small attenuated flagellates measuring from 6μ to 8μ in length and 2μ to 3μ in breadth. Their protoplasm stained dark blue but contained no vacuoles or granules; the macronuclei were circular or oval in shape occupying nearly the whole of the centre of the parasite. In some of tne cells the macronuclei showed evidences of further division, having a bilobed appearance, each lobe containing a number of chromatic rods (Plate II, fig. 5 *a*). The micronuclei in the majority of the cells lay close up to the macronuclei and the flagella were comparatively short, measuring from 8μ to 12μ in length.

These bodies were attached to one another either by their posterior poles or along the whole length of the body, and it is evident that they had originated from the further longitudinal division of the oval parasites which result from the division of the spindle-shaped ones. I was unable to satisfy myself as to whether these small parasites may have been produced from the long flagellates seen in Plate II, fig. 4, as the majority of these forms showed no evidences of longitudinal division.

Plate II, fig. 5 *b*, shows a large oval flagellate measuring 8·5μ in diameter with a smaller parasite splitting off from its side. The macronucleus of the parent cell occupies its posterior pole and shows signs of division. The free portion of the flagellum of the large cell is considerably thickened almost to its end, while the attached part is divided into two filaments which pass into a large flagellar vacuole just anterior to the micronucleus. The macronucleus of the smaller cell is also situated at the posterior pole and its micronucleus lies close up to it. Its flagellum is almost separated from that of the parent cell being attached by its end alone. The commencing division of the flagellum and macronucleus of the parent cell and the position of its micronucleus show that it is about to divide longitudinally.

On January 2nd the remaining bugs, three adult males and one female, were dissected between 12 noon and 1-30 P.M., the midguts being dissected out and prepared as described above; in addition smears were made from the crops and salivary glands of all the bugs. In two films, one prepared from the midgut of a male and the other from that of a female, there were a number of parasites, some exhibiting the earliest changes towards development, others more advanced, as well as a few rosettes and small flagellates.

Plate II, fig. 5 *e* and *f*, shows two parasites in the early stages of development; in the smaller the flagellar vacuole is seen as a small pink area lying near the micronucleus. The larger parasite measures 8μ in length and 4·5μ in breadth, one end being rounded and the other pointed; its protoplasm stained dark blue with Giemsa's stain and contained some small pale areas, but none of the distinct vacuoles usually seen at this stage. Its micronucleus a little enlarged lay close to the macronucleus which is round and compact staining deep red.

Plate II, fig. 6 *a*, shows another cell of a similar nature, though it is almost round; its protoplasm is less deeply stained and contains a few indistinct vacuoles. Its macronucleus lying near one end is almost divided into two, while the micronucleus is situated about the centre and lying near it is a faint pink mass suggesting the early development of a flagellum. These parasites clearly show that the Leishman-Donovan body may remain in the midgut of the bug for at least five days before they begin to develop.

The rosettes seen in these slides are similar to those described above, the majority consisting of fully developed flagellates. Plate II, fig. 5 *c*, shows a long flagellate which measures 9·5μ in length and 2·5μ in breadth; its oval macronucleus is situated about the centre of its body and lying close to it and about 1·5μ from the anterior end is the micronucleus. The posterior end of the parasite is pointed and situated about 3μ from this end there are two small pink masses which on higher magnification are seen to consist of a number of chromatic granules. This is the first parasite that has shown this appearance, though in another long flagellate (Plate II, fig. 4) there was a suggestion of the same structure. From these isolated specimens it is impossible to say what this represents, but further reference will be made to it later.

Plate II, fig. 5 *d*, represents two attenuated flagellates attached by their poles, and from the outer side of one of the cells a spirilla-like parasite is seen splitting off. The macronuclei of the two parasites are circular and compact staining uniformally dark red or almost black with Giemsa's stain; in one cell the macronucleus is near the anterior end and in the other towards the posterior pole, and in each case the micronuclei are lying close up to their respective macronuclei. The protoplasm of these cells stains dark throughout and is devoid of granules or vacuoles. The spirilla-like parasite is 5·4μ in length and 1μ in breadth, and in its protoplasm there are two small chromatic granules, the anterior being distinctly rod shaped.

These pairs of attenuated flagellates are seen in all stages of longitudinal division; some are without flagella and are similar to those described and figured in my first report which therefore represent a very late stage in the development of the parasite. Plate II, fig. 6 *c*, represents one of these attenuated flagellates lying free; it measures 9μ in length and 1·2μ in breadth, and its flagellum measures 10μ. The macronucleus, which consists of a number of chromatic rods, is situated about the centre, while the micronucleus is 2μ from the anterior end. The protoplasm of the cell appears very granular and stains deep blue with

Giemsa's stain. The flagellum, a stout filament, is inserted into the body near the micronucleus, though not attached to it.

Another type of small flagellate dividing longitudinally is shown in Plate II, fig. 6 *b*. Its two daughter cells further show evidences of multiple division of their macro-and micronuclei. In one of these there are two macronuclei, one circular in shape situated at the posterior pole, the other having a dumb-bell appearance situated about the centre. There are also three micronuclei in this cell, two near the central macronucleus and the other towards the posterior end. In the second cell there is a single macronucleus lying about the centre with the micronucleus near it. Two flagella are seen passing out from one cell and one from the other. Fig. 6 *d* shows two flagellates in each of which there are two macronuclei, two micronuclei and a single flagellum.

The process of irregular division in the larger oval forms undoubtedly results in the formation of small irregularly shaped flagellates shown in Plate II, fig. 6 *e* and *f*; one of these small cells shows a dumb bell shaped macronucleus and three micronuclei. Its posterior end is distinctly pointed while the rest of the cell is rounded. Fig. 6 *f* represents two small flagellates attached by their posterior poles in which the round macronuclei are lying with the two micronuclei near them. The protoplasm of all these small cells stains uniformly dark blue with Giemsa's stains and contains heither vacuoles nor granules.

Plate II, fig. 6 *g*, shows a large flagellate measuring 15μ in length; two macronuclei are seen lying on each side of the centre of its body and a little posterior to one there is a single chromatic dot. One micronucleus is 2μ from the anterior end and the other about 5μ, and the flagellum is inserted near the anterior one. Two chromatic filaments are seen extending from the vicinity of the macronuclei, and uniting lower down pass into the flagellum. This was the only flagellate exhibiting these appearances, and later when discussing the development of the parasite reference will again be made to it. I was unable to find any parasites in the smears made from the crops and salivary glands of the bugs.

There can be no doubt from the above description that the Leishman-Donovan bodies had undergone marked developmental changes in the bed-bug (*Cimex rotundatus*), the following being a summary of the nature and order of sequence of these changes. On the second day after being ingested by the bug the parasites begin to develop, their protoplasm increasing in volume; at the same time the macronuclei enlarge and show signs of commencing division. The parasites may now either pass on to flagellation, or further growth followed by the consecutive division of the macro-and micronuclei may result in the formation of rosettes. The single flagellates enlarge and begin to divide longitudinally to result in oval or spindle-shaped cells; while the rosettes after flagellation begin to divide up into separate elongated flagellates. The oval or spindle-shaped parasites divide repeatedly either by equal or unequal longitudinal division and result in smaller and more irregular forms. All these changes may be seen in the bug during the first three days. Still later longitudinal division of the oval and irregular flagellates progresses rapidly, so that by the fifth day the majority have become small or spirilla-like flagellates.

As the bugs were dissected at intervals of about 24 hours it was difficult to follow some of the later stages of the development. For instance, it is probable many spindle-shaped or oval cells are produced directly from the rosettes, and further the long fully developed flagellates may be formed from these spindle-shaped cells. I was also unable to find any evidence to show that the long flagellates may divide longitudinally, and none of them had the spirilla-like forms splitting off as described by Leishman.

The small irregular flagellates containing more than one macro-and micronucleus figured in Plate II, fig. 6, result most probably from the rapid and irregular division of the oval forms produced from the spindle-shaped parasites. Further, it must be remembered that the bugs had not fed for five days, so that it is quite possible some of the later changes were considerably modified by the absence of blood.

During the six days the parasites were developing the bugs were kept in an open verandah where the average temperature was 72°F. From the fact that in cultures the parasites only develop below a temperature of 75°F, Rogers[13] believes that the majority of patients become infected with the Leishman-Donovan body during the cold season. He finds from an analysis of two series of cases in Calcutta, that the disease commenced during the cold or early in the hot weather. Dr. Dodds Price[13] also states that in his European cases and in the majority of his native patients the disease began during the cold weather. From these observations it appears that the disease usually begins in the cold season, and if, as Rogers states, the incubation period is probably a long one, it is natural to conclude that the patients were infected months earlier in the hot season.

As stated in my first report[1] I found that the parasites developed into flagellates when the temperature was between 80° and 82° F. ; and as we do not know even the approximate length of the incubation period, it seems premature to conclude that the infection can take place only during the cold season. A large number of experiments with infected bugs kept at different temperatures are necessary before we can hope to solve this point.

On comparing the development of the parasite in the bug with that seen in cultures *in vitro* as described by Rogers,[15] Christophers,[13] Leishman and Statham,[14] it will be seen that though they agree in many points, they differ considerably in others. Vacuolation of the protoplasm in the earlier stages of development is seen in both the bug and in the cultures, but it was absent in the later states in the development in the bug. The chromatic dots which Leishman has described occuring in pairs in the protoplasm both in the younger stages prior to exflagellation as well as in the mature parasites were not seen in any of my films. Christophers and Leishman have described the formation of two or more parasites resulting from the consecutive division of the macro-and micronucleus of a single cell, and the latter mentions that these cells may give rise to three or even four individuals by a process of further sub-division of the macro-and micronuclei. This process is apparently a marked feature of the development in the bug resulting in the formation of rosettes of from four to eight flagellates.

In the bug longitudinal division of the round or oval parasites was not seen prior to flagellation, though in some of the parasites the macronuclei showed evidences of division; all the twin parasites had grown to maturity while lying side by side. This method of multiplication is therefore entirely limited to the flagellate stage.

In the majority of the spindle-shaped and oval flagellates the micronuclei were seen close up to the macronuclei, seldom at the anterior end; in the long mature flagellates and spirilla-like forms, however, the micronuclei were situated some distance from the macronuclei and about 1·5μ to 2μ from the anterior ends. In the case of *Trypanosoma lewisi* when a parasite is about to divide the blepharoplast passes close up to the nucleus, only resuming its final position when the cell assumes its adult form. When this position of the micronucleus is seen in a young flagellate of the Leishman-Donovan body, it indicates that the cell has not yet reached maturity and that further division is about to take place. In my first report I drew attention to a few instances of parasites showing this appearance, but I was not then able to explain its significance.

A number of attenuated parasites were seen free, and in one case a thin cell containing two chromatic dots was still attached to a small flagellate. Multiple division of the macro-and micronuclei of the small oval flagellates takes place in the bug about the fifth day resulting in the formation of still smaller flagellates which are themselves capable of further longitudinal fission. So far as I am aware, this process has not been seen in cultures. In the later stages of the development of the parasite in the bug two distinct types of flagellates were seen; a long relatively thick form measuring from 9μ to 15μ in length and 2μ to 3μ in breadth, and a small irregular flagellate measuring from 4μ to 6μ in length and 1μ to 4μ in breadth. In both the micronuclei are situated about 2μ from the anterior ends through the centre of which the flagella pass out.

There was no evidence to show that the thin parasites originated from the larger ones, though Leishman has observed this process in cultures, and further, as no sexual cycle was seen, it is not possible to say whether they represent two distinct sexes. In one of the large flagellates (Plate II, fig. 5 *c*) two pink areas containing a number of chromatic granules were seen; it is probable they represent the diplosome observed in other flagellates. In another parasite (Plate II, fig. 6 *g*) there was a suggestion of a similar structure at the posterior end, and from its vicinity a chromatic thread passed forwards and joining a similar filament terminated in the flagellum. In *Herpetomonas muscæ domesticæ* Prowazek[19] describes a spiral thread which passes to the posterior end of the parasite and terminates in a diplosome; prior to the process of copulation the diplosome divides resulting in a group of six granules. As these appearances were seen only in a few of the parasites, it was not possible to follow the further changes, if any, undergone by them.

It is hoped later to direct special attention to the final stages of the development of the parasites and their method of re-entry into the human body.

The Bed-Bug.

The fact that the Leishman-Donovan body undergoes its extracorporeal development in the bed-bug, *Cimex rotundatus*, makes it necessary to describe briefly the two species of this insect associated with man.

According to Mr. Distant[20] the family *Cimicidæ* which contains four genera, *Cimex*, *Oeciacus*, *Cacodmus* and *Hematosiphon*, belongs to the *Heteroptera*, a sub-order of the *Rhynchota*, and is placed in the series *Gymnocerta* between the families *Phymatidæ* and *Ceratocombidæ*. Mr. Distant informs me the genus *Cimex* at present contains four species, *Cimex lectularius* Linn., *Cimex rotundatus* Sign, *Cimex pipistrelli* Jenyns[21], and *Cimex columbarius* Jenyns[21], all of which have the following characters: The head is short and broad with two prominent eyes, but no ocelli. The antennæ are four jointed, the apical joints being slender; the elytra are rudimentary and lie over the metathorax. The prothorax is semilunar in shape with its anterior angles considerably extended. The abdomen, which is uncovered, consists of seven segments and an eighth anal appendage; the legs are slender, the anterior tibiæ more than twice as long, and the posterior three times as long, as the tarsi, which are three jointed. The proboscis is flexed in a groove beneath the head and prothorax.

Type species Cimex lectularius Linn. The adult insect of this species is reddish brown in colour and is covered with fine hairs; it varies in length from 4·5 to 5 millimeters, the male usually being a little smaller than the female. The head is short and broad and is inserted into a notch in the prothorax; it contains two lateral prominent eyes in front of which are the two antennæ and in the median line the thick first segment of the labrum which is continuous with the dorsal integument of the head. The antennæ consist of four segments, the first two being the thicker, while the third and fourth are slender and covered with long hairs.

The prothorax is semilunar in shape with two rounded horns extending close up to the eyes; its upper surface is raised in the centre and towards the sides, ending abruptly at a line a little beyond the level of the eyes; the remainder of the surface including the two horns is flattened from above downwards. The ventral surface of the prothorax is concave and hollowed out on each side of the mid-line where the first pair of legs are inserted. The mesothorax as seen from the dorsal surface is triangular in shape with its apex projecting over the metathorax and between the elytra. The metathorax next in size to the prothorax is almost entirely covered on its dorsal side by the elytra, and on the ventral surface is seen as a small cleft on the inner side of the middle coxa. The elytra which are inserted into the mesothorax just below the lateral angles are two rudimentary scallop-shaped pieces of chitin lying over the metathorax and sides of the first abdominal segment. Their dorsal surfaces are convex and covered with bristles, while their ventral surfaces are concave. The abdomen is rounded and consists of seven segments with an eighth anal appendage. It is broadest at the third segment and gradually becomes narrower towards the end where it is covered with long hairs. In the male the penis is seen flexed in a notch between the seventh and eighth segments.

This bug is distributed throughout Europe and North America, and is also found in Suez, Egypt, the Sudan, the North-West Frontier Province of India, China, South Africa and Australia.

Cimex rotundatus Sign or the Indian bed-bug is darker than the above species being of deep mahogany colour; its head is not as long or as broad as that of *lectularius.* Its prothorax is also narrower and shorter, is more rounded and not flattened at the sides as is the prothorax of the type species. Its abdomen is less orbicular, being broadest at the second segment and tapers more abruptly towards the end; in all other respects it is similar to *Cimex lectularius.*

Cimex rotundatus is distributed throughout India, Burma, Assam, Malay, and is also found in Aden, Sierra Leone, the Islands of Mauritius, Reunion, St. Vincent and Porto Rico.

Cimex pipistrelli Jenyns is similar in colour to *rotundatus,* its prothorax is also less marginal and in all other respects it is more closely allied to the Indian bed-bug.

Cimex columbarius Jenyns has a similar prothorax to *lectularius* and is also of the same colour.

Dissection of the bed-bug. The following method of dissecting the bed-bug has been found satisfactory. After having killed the insect by placing it in a tube with a plug of cotton wool containing a few drops of chloroform, it is taken up with a fine pair of forceps and the legs are pulled off. In removing the fore legs it is necessary to be careful not to injure the prothorax, as they are firmly fixed in concavities on its under surface. The elytra are next removed by raising them with a fine pair of forceps and gently twisting them off from their joints at the angles of the mesothorax. The bug is now placed in a drop of normal saline solution with its head directed towards the dissector. A fine needle is inserted with the left hand into the right side of the prothorax, while with another fine needle in the right hand the joint between the prothorax and mesothorax is separated. By gently drawing the needle in the left hand and by exerting pressure on the dorsal surface of the abdomen with the other needle, the œsophagus, midgut and the remaining part of the intestinal tract are drawn out, and any portion can then be isolated and examined. The salivary glands lying on each side of the œsophagus are also exposed by this method. The ducts of the salivary glands and the œsophagus can be followed into the crop situated in the head by carefully removing the prothorax.

Conclusions.

1. Though Kala Azar is a chronic disease lasting many months and often years, it occasionally runs an acute course terminating in from four to five months as illustrated by the case described above. In these acute cases as well as in the chronic ones terminating with ulceration of the large intestine, the parasites are found a few days before death in large numbers in the peripheral blood in the leucocytes and endothelial cells, and the latter are probably the source of all the parasites seen in the circulating blood.

2. Though the parasites are most abundant in the peripheral blood towards the end of the disease, they are also found in the early stages as a case recently seen clearly illustrated.

3. In the female as well as in the male bed-bug (*Cimex rotundatus*) the parasites have by the third day passed through all the intermediate stages of development described above up to the formation of the mature flagellates. Rapid multiplication by rosette formation is a characteristic feature of the development of the parasite in the bed-bug. As the male bug sucks blood it probably plays as important a rôle in the transmission of the disease as the female bug.

4. The infection acquired by the bug varies considerably, some ingesting large numbers of parasites, others only a few; and there is no evidence at present to show that the development in the bug depends on variations in the temperature.

5. The tendency that the disease has to linger in a house for a long time is probably explained by the fact that the parasite may remain in the midgut of the bug for several days before beginning to develop, and, as the nymphs which take from seven to ten weeks to arrive at maturity, may ingest the parasites shortly after hatching, and as a rule feed only once between each moult, the infection may remain for a considerable time in a house ; there is no evidence at present to support the view that the infection is inherited by the bug.

I wish to take this opportunity of thanking Mr. Lounsbury for specimens of *Cimex lectularius* L. from South Africa, Mr. W. Rainbow, Australian Museum, for specimens of *Cimex lectularius* from Sydney, Dr. C. W. Branch for a large collection of *Cimex rotundatus* Sign. from the Island of St. Vincent, Dr. J. W. W. Stephens for specimens of *Cimex rotundatus* from Sierra Leone, Dr. C. A. Butler, United States Naval Hospital, San Juan, for specimens of *Cimex rotundatus*, and Mr. W. L. Distant for information and advice.

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G. I. C. P. O.—No. 1 S.C.—11-9-07.—1130.

EXPLANATION OF PLATE I.

Figure 1.—Polymorphonuclear leucocyte containing six parasites. Stained by Romanowsky's stain. Magnification 550 diameters.

Figure 2.—Endothelial cell containing forty-nine parasites. Stained by Romanowsky's stain. Magnification 550 diameters.

Figure 3.—Eosinophyl leucocyte containing two parasites. Stained by Romanowsky's stain. Magnification 550 diameters.

Figure 4.—Endothelial cell containing thirty-two parasites, six of which are lying on the nucleus. Stained by Romanowsky's stain. Magnification 550 diameters.

Figure 5.—Large blue body containing two parasites from the peripheral blood of patient mentioned in text. Stained by Romanowsky's stain. Magnification 550 diameters.

Figure 6.—Large mononuclear leucocyte much distorted containing ten parasites; note two of the parasites show commencing development. Stained by Romanowsky's stain. Magnification 550 diameters.

Figure 7.—Parasites showing the early stages of development. Stained by Giemsa's stain. Magnification 1,000 diameters.

(*a*) Two unchanged parasites lying in a disintegrating leucocyte 80 hours after being ingested.

(*b*) Parasite with a large central vacuole lying in a degenerating leucocyte; the macronucleus shows commencing enlargement.

(*c*) Five parasites lying grouped together in the remains of a leucocyte; four of the cells are much enlarged, their protoplasm is vacuolated, the flagellar vacuoles are well developed, and the macronuclei show evidences of commencing division; the fifth parasite is almost unchanged.

(*d*) Enlarged parasite lying in a leucocyte, the macronucleus is granular and situated across the cell; the protoplasm stains dark blue and contains a number of large circular vacuoles.

(*e*) A similar parasite.

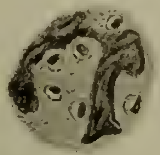
(*f*) Much enlarged parasite with deep blue granular protoplasm and large circular vacuoles; the flagellum is commencing to form.

Figure 8.—Parasites showing the early formation of the flagellum. Stained by Giemsa's stain. Magnification 1,000 diameters.

(*a*) Enlarged parasite with an elongated macronucleus surrounded by groups of vacuoles. Adjacent to the micronucleus is the circular flagellar vacuole which consists of dark strands lying in a light pink matrix.

(*b*) Enlarged oval parasite with two micronuclei close to which is the developing flagellum.

(*c*) Parasite showing the very earliest appearance of the flagellum which is seen as a light pink area with a darker centre lying in the blue staining protoplasm.



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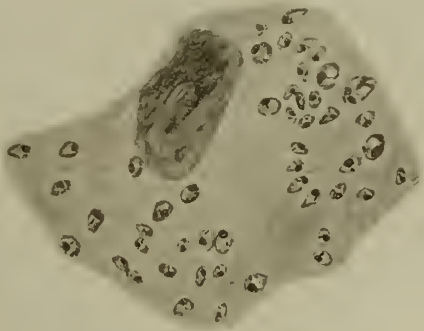

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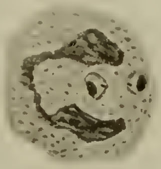

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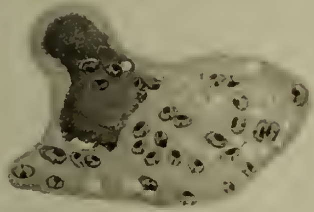

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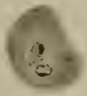

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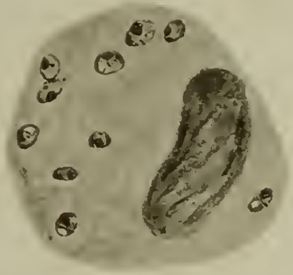

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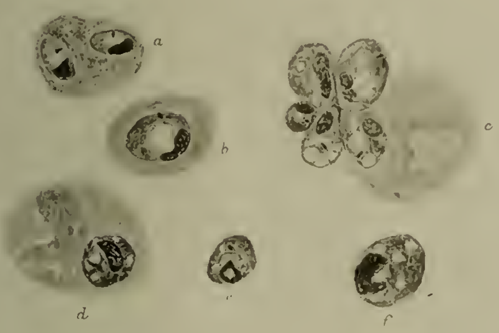

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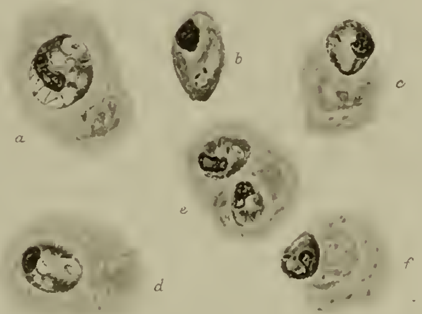

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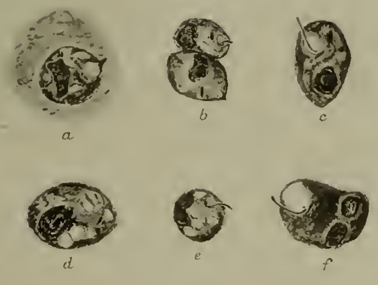

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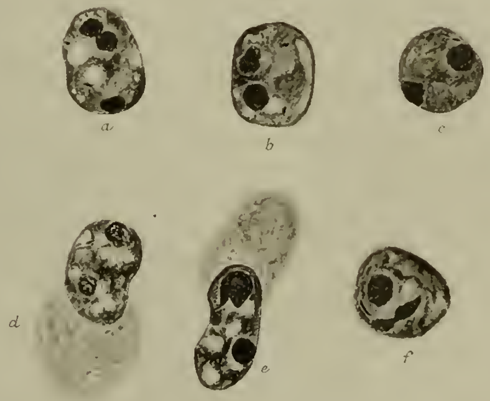

Fig. 10.

Photogravure.

Survey of India Offices, Calcutta, November, 1907.

(*d*) Parasite very similar to (*a*), the micronucleus is thickening prior to division.

(*e*) Two parasites showing the early development of the flagellum; in one there are two micronuclei.

( ) A similar parasite, the macronucleus shows evidences of division.

Figure 9.—Parasites showing the early protrusion and growth of the flagellum. Stained by Giemsa's stain. Magnification 1,000 diameters.

(*a*) Parasite showing the flagellum protruding as a short thick brush of fine filaments.

(*b*) Twin parasites showing the early protrusion of the flagellum; in one it can be traced into the vacuole to a point close to the micronucleus.

(*c*) Parasite showing the flagellum passing into the vacuole to a point in close proximity to the micronucleus.

(*d*) Large vacuolated parasite showing the flagellar vacuole passing to the margin prior to rupture.

(*e*) Circular parasite with flagellum seen as a short curved rod; the macronucleus is dumb-bell shaped and is about to divide.

(*f*) Large parasite with short flagellum which can be traced up to a chromatic dot close to the micronucleus; the macronucleus has just divided.

Figure 10.—Parasites showing the method of formation of the true rosette. Stained by Giemsa's stain. Magnification 1,000 diameters.

(*a*) Large oval cell consisting of deep blue granular protoplasm containing a number of small circular vacuoles. The original macronucleus has divided and one of the daughter nuclei is about to divide; associated with the three micronuclei there are three large flagellar vacuoles.

(*b*) Large parasite with two macro-and micronuclei, the former show signs of commencing division; there are no flagellar vacuoles.

(*c*) A similar cell; note that the macronuclei are situated at opposite poles.

(*d*) Large vacuolated parasite showing the recent division and commencing separation of the micronuclei.

(*e*) Elongated parasite, one of the daughter nuclei is commencing to divide. The two micronuclei have already divided.

(*f*) Oval cell showing division of a daughter nucleus; the micronucleus is thickened and elongated prior to transverse division.

EXPLANATION OF PLATE II.

Figure 1.—Enlarged parasites showing longitudinal division and multiple segmentation. Stained by Giemsa's stain. Magnification 1,000 diameters.

- (*a*) Large bean-shaped parasite with a fully developed flagellum which ends in a chromatic dot between the macro- and micronucleus.
- (*b*) Similar parasite showing longitudinal division into two smaller flagellates. Note as division has taken place before the flagellum has fully developed, it has split throughout its length.
- (*c*) Two large parasites showing various stages of longitudinal division.
- (*d*) Rosette of four flagellates showing the division of the protoplasm.
- (*e*) Large rosette containing five macronuclei and six micronuclei; four flagella have passed out from one side and a fifth is developing in the centre.

Figure 2.—Spindle-shaped flagellates. Stained by Giemsa's stain. Magnification 1,000 diameters.

- (*a*) Spindle-shaped flagellate dividing, the second flagellum has split off from only a part of the original one.
- (*b*) Similar cell showing unequal longitudinal division.
- (*c*) Two long parasites formed by the division of a large spindle-shaped flagellate.
- (*d*) Division of a large spindle-shaped flagellate into two smaller ones.
- (*e*) Two similar parasites; the micronuclei are still close up to the macronuclei and are elongating indicating the cells are about to divide.

Figure 3.—The rosette and its division. Stained by Giemsa's stain. Magnification 1,000 diameters.

- (*a*) Two large flagellates detached from a rosette containing four parasites.
- (*b*) Four elongated flagellates from a rosette of six parasites.
- (*c*) Large rosette containing two large nuclear masses, eight micronuclei and six flagella. In these rosettes the majority of the flagella usually pass out fron one side of the cell.
- (*d*) Two long flagellates from a rosette of six parasites.

Figure 4.—Four long flagellates, in one there are a few granules at the posterior end. Stained by Giemsa's stain. Magnification 1,000 diameters.

Figure 5.—Appearances seen towards the end of development. Stained by Giemsa's stain. Magnification 1,000 diameters.

- (*a*) Two small flagellates attached by their posterior poles; one of the cells is about to divide again.
- (*b*) Large oval flagellate with a smaller cell splitting off.
- (*c*) Small elongated flagellate with two chromatic granules at the posterior end.
- (*d*) Two attenuated flagellates from the outer side of one of which a thin parasite is splitting off.

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Plate II.

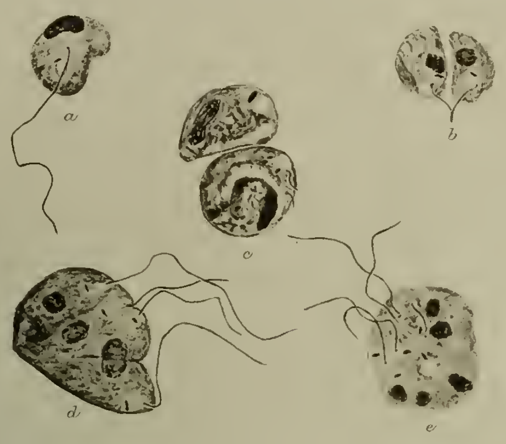
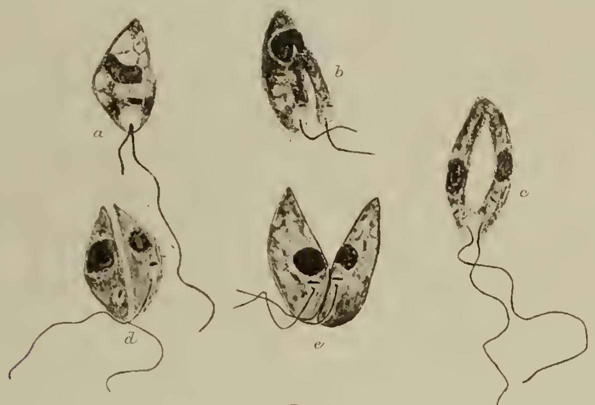


Fig 1.



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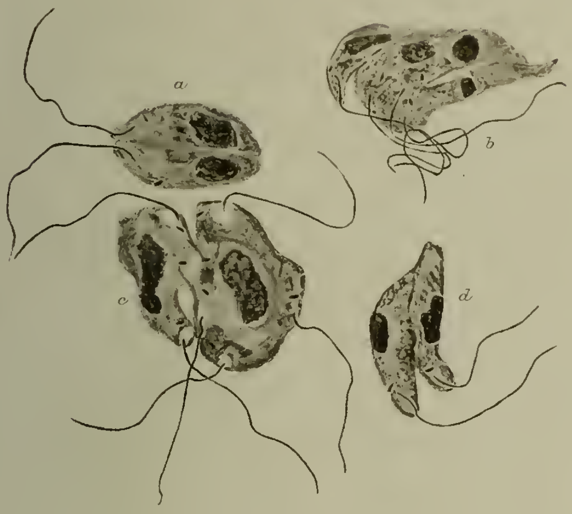

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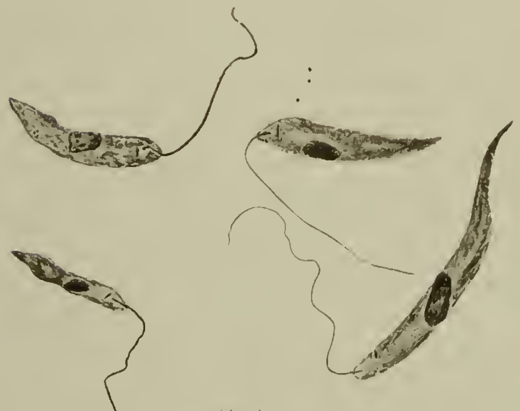

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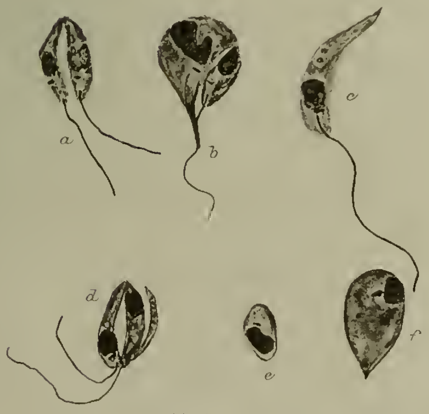

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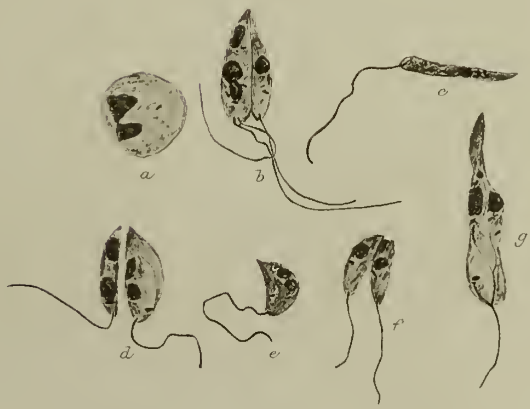

Fig. 6.

Photogravure.

Survey of India Offices, Calcutta, September 1907

(*e*) Small oval parasite showing the early formation of the flagellar vacuole; this parasite had been in the midgut of the bug for five days.

(*f*) Larger oval parasite from the same bug. There was no appearance suggesting the development of the flagellum.

Figure 6.—Flagellates showing multiple segmentation. Stained by Giemsa's stain. Magnification 1 000 diameters.

(*a*) Enlarged sound parasite showing the develpment of the flagellum recovered from a bug five days after being ingested.

(*b*) Small flagellate showing multiple division which results in the formation of small irregular flagellates.

(*c*) Free attenuated flagellate.

(*d*) Two small flagellates, the result of the division of a larger oval or spindle-shaped cell; each daughter cell shows evidences of multiple segmentation.

(*e*) Small irregular flagellate containing two macronuclei and three micronuclei.

(*f*) Two small flagellates attached by their posterior poles

(*g*) Long flagellate showing evidences of irregular division.

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THE DEVELOPMENT OF THE LEISHMAN-DONOVAN PARASITE IN CIMEX ROTUNDATUS

SECOND REPORT

BY

CAPTAIN W. S. PATTON, M.B., I.M.S.
(*On special duty.*)

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